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# Preventive Effect of Sugars on Denaturation of Fish Protein during Frozen Storage

Zentaro OOSHIRO*, Yasumasa HIRONAKA* and Seiichi HAYASHI*

**Abstract**

The cryoprotective effect of glucose was increased in proportion to the concentration of glucose. Maltotriose was most effective on ATPase activity, protection of SH-group and solubility of actomyosin during frozen strage.

It was found that hydroxyl group of sugars was an important factor for preventive effect of cryoprotectant, and that the structure of sugars was also an important factor.

The preventive effects of sorbitol and glucose on the denaturation of carp muscle actomyosin during frozen storage were reported by TAKASHI *et al*,[1] and the effects of some amino acids, peptides, acetyl amino acids, carboxylic acids and carbohydrates were investigated and discussed availably by NOGUCHI.[2] He summerized the property of cryoprotectants from view point of molecular structure.

This report describes about the preventive effects of derivatives of glucose and about the different effects due to the difference in molecular weight, number of polar group and structure of molecules of sugar.

## Experimental Procedure

**Preparation of actomyosin from carp muscle.** An alive breeding carp, *Cyprinus carpis*, about 750 g of body weight and about 25 cm of body length, was killed instantly by electric shock in a refrigerator at about 2°C. Immediately the muscle was cut off and minced with a meat grinder. Then the actomyosin was prepared according to procedure of Table 1. All operations were performed at about 4°C.

**Procedure of frozen storage of carp muscle protein.** The protein solution which was prepared according to the Table 1 was diluted to the concentration of 3–5 mg protein/m*l*. with 0.6 M KCl, and this solution was the sample for freezing storage experiment. The definite volume of additives was added to the sample, the solution was carefully stirred to avoid bubbling at about 2°C for 16 hr. At the same time, additive free sample as a control was also carried out according to the same procedure. The aliquot of 15 m*l* of these samples was placed respectively into test tubes, stored in a deep freezer at −20°C for various periods, namely 2, 4 and

* Laboratory of Food Chemistry, Faculty of Fisheries, University of Kagoshima.

Table 1. Procedure of the preparation of carp actomyosin.

| |
|---|
| Coarsely minced muscle of carp (50 g) |
| —— washed 2 times with phosphate buffer (pH 7.5, I=0.05) |
| —— centrifuged at 5000 G for 20 min. |
| Residue |
| —— extracted with 5 volumes of 0.45 M KCl-phosphate buffer (pH 7.5) |
| —— centrifuged at 5000 G for 20 min. |
| Supernatant |
| —— diluted slowly twice with 10 volumes of cold water |
| —— centrifuged at 5000 G for 20 min. |
| Precipitate |
| —— dissolved in 1.2 M KCl with equal volume of the precipitate |
| —— stirred for 3 hr with magnetic stirrer |
| —— centrifuged at 15000 G for 30 min. |
| Supernatant (crude actomyosin) |

8 weeks. After the storage for appropriate periods, these samples were thawed at room temperature, and the solubility, ATPase activity, the number of thiol group and the viscosity of the protein were measured. They were given as indicator of protein denaturation.

**Determination of ATPase activity.** Assay mixture contained 2 m*l* of 62.5 mM Tris-maleate buffer (pH 6.8), 2 m*l* of 12.5 mM $CaCl_2$ 0.5 m*l* of 10 mM ATP and 0.5 m*l* of actomyosin suspension. The enzyme reaction was initiated by the addition of ATP at 25°C, stopped by the addition of 2 m*l* of 15% perchloric acid and filtered with filter paper. The inorganic phosphate in the filtrate was determined by the method of ROCKSTEIN *et al.*[3] One enzyme unit was defined as the amount of enzyme that catalized the release of one $\mu$mole of inorganic phosphate under the assay conditions per minute.

**Determination of residual thiol group.** Thiol group of actomyosin was determined by the method described by MATSUMOTO.[4]

**Determination of viscosity.** Viscosity of actomyosin was measured by the use of viscometer, Ostwald's viscometer.

**Determination of N-acetyl-D-glucosamine.** N-acetyl-D-glucosamine was determined by the method described by FUKUI.[5]

**Determination of hydrophobic area of protein.** Hydrophobic area of protein was determined by using the fluorescent pigment, 1-anilinonaphthaleno-8-sulfate (ANS) at the concentration of 25 $\mu$g per m*l* of water. The intensity of fluorescent of standard solution (9 m*l* of n-propanol+1 m*l* of fluorescent pigment solution) was adjusted to 100%. Then the intensity of fluorescence of the sample (1 m*l* of protein solution+8 m*l* of 0.6 M KCl, pH 6.8+1 m*l* of fluorescent pigment solution) was read.

**Paper chromatography.** Components of polymers of glucose were analyzed and identified by paper chromatography using the solvent system; pyridine: n-butanol: water=4: 6: 3. After development, papers were sprayed to detect components of polymers with the solvent; 1.7 g phthalate and 0.93 g aniline in 100 m*l* of n-butanol saturated with water.

**Determination of glucose.** Glucose was determined by using anthrone reagent.[6]

**Determination of protein.** Protein was determined from the absorbance at 280 m$\mu$.

**Preparation of polymers of glucose.** As polymers of glucose obtained as commercial reagents were dimer and trimer, polymers composed of more than four molecules of glucose was prepared from the partial decomposition of starch by $\alpha$-amylase and $\beta$-amylase, which was a kind gift from Nippon Starch Co., Ltd. The separation of polymers of glucose was carried out by adsorption chromatography with activated charcoal.

**Preparation of 1, 2, 6-acetyl-D-glucosamine.** The mixture of 50 g of acetic anhydride and 50 g of pyridine anhydride was cooled at 0°C and 10 g of N-acetyl-D-glucosamine was added to the mixture. The mixture was allowed to stand at room temperature for 2 or 3 days. The reactant was added to ice-water (200 m*l*) with stirring contineously and extracted 3 times with 150 m*l* of chloroform. The extractant was washed successively 3 times with 150 m*l* of 1 N HCl, and 2 times with 150 m*l* of water, and dried over anhydrous sodium sulfate. The solution was concentrated under reduced pressure with rotary evaporator. The concentrated was dissolved in a few volume of hot alcohol. Ether was added to the solution and the crystal was deposited slowly. The crystal was recrystallized from same solvent. The amount of recovery was 61%. The melting point of the crystal was from 138 to 139°C and $[\alpha]_D^{16}$ was +90°.

**Preparation of $\alpha$-pentaacetyl-D-glucose.** The mixture of 50 g of acetic anhydride and 65 g of pyridine anhydride was cooled at 0°C. Glucose (10 g) was added to the mixture and the mixture was stirred until glucose was dissolved completely. Then it was allowed to stand at room temperature for 18 hr. The deposited crystal was recrystallized from 95% ethyl alcohol. The yield of product was 19 g. The melting point of the crystal was from 112 to 113°C and $[\alpha]_D^{16}$ was +102°.

## Results

**Effect of the concentration of glucose.** In order to clarify the preventive effect of the glucose added to the actomyosin solution at various concentration, glucose concentration was varied as follows; 0.2, 2.0, 20.0 and 200.0 mM. These solutions of protein containing glucose were frozen at −20°C and stored for 2, 4 and 8 weeks. After thawing at room temperature, solubility, ATPase activity, numbers of thiol group and reduced viscosity of protein were determined. Results were shown in Table 2.

Table 2. Effect of the concentration of glucose on the prevention of denaturation of actomyosin.

Protein Solubility

| Glucose mM | Weeks 0 | 2 | 4 | 8 |
|---|---|---|---|---|
| 0 | 100% | 47.7 | 37.2 | 30.2 |
| 0.2 | | 48.2 | 37.9 | 32.3 |
| 2.0 | | 49.4 | 39.8 | 36.9 |
| 20.0 | | 58.1 | 48.5 | 48.5 |
| 200.0 | | 62.1 | 54.4 | 47.1 |

ATPase Activity

| Glucose mM | Weeks 0 | 2 | 4 | 8 |
|---|---|---|---|---|
| 0 | 100% | 67.2 | 51.8 | 22.6 |
| 0.2 | | 75.4 | 64.3 | 33.5 |
| 2.0 | | 78.8 | 78.4 | 46.3 |
| 20.0 | | 76.2 | 104.8 | 94.8 |
| 200.0 | | 91.0 | 115.3 | — |

Numbers of SH-Group

| Glucose mM | Weeks 0 | 2 | 4 | 8 |
|---|---|---|---|---|
| 0 | 100 % | 91.0 | 91.3 | 78.5 |
| 0.2 | | 96.5 | 91.3 | 66.6 |
| 2.0 | | 97.0 | 80.2 | 83.6 |
| 20.0 | | 108.6 | 97.1 | 91.5 |
| 200.0 | | 140.6 | 99.3 | — |

Reduced Viscosity

| Glucose mM | Weeks 0 | 2 | 4 | 8 |
|---|---|---|---|---|
| 0 | 0.67 | 0.29 | 0.25 | 0.12 |
| 0.2 | 0.68 | 0.32 | 0.27 | 0.18 |
| 2.0 | 0.67 | 0.33 | 0.30 | 0.23 |
| 20.0 | 0.69 | 0.33 | 0.39 | 0.31 |
| 200.0 | 0.77 | 0.35 | 0.39 | 0.36 |

Table 3. Effect of monomer, dimer and polymers of glucose on the prevention of denaturation of actomyosin.

Protein Solubility

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 100% | 56.8 | 48.3 | 29.2 |
| glucose | | 67.7 | 65.0 | 54.0 |
| maltose | | 67.3 | 66.7 | 54.0 |
| maltotriose | | 67.7 | 69.0 | 58.6 |
| maltotetrose | | 64.6 | 67.9 | 57.6 |
| maltopentose | | 67.0 | 66.7 | 52.3 |

ATPase Activity

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 100% | 71.1 | 48.2 | 35.9 |
| glucose | | 107.0 | 97.8 | 102.5 |
| maltose | | 106.1 | 83.0 | 96.4 |
| maltotriose | | 108.9 | 90.2 | 108.9 |
| maltotetrose | | 85.8 | 79.0 | 94.4 |
| maltopentose | | 102.4 | 83.0 | 95.4 |

Numbers of SH-group

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 100% | 85.7 | 71.3 | 64.1 |
| glucose | | 84.8 | 78.4 | 71.1 |
| maltose | | 87.7 | 89.5 | 69.8 |
| maltotriose | | 91.3 | 84.5 | 70.3 |
| maltotetrose | | 79.1 | 73.5 | 71.1 |
| maltopentose | | 72.8 | 70.4 | 65.7 |

Reduced Viscosity

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 0.77 | 0.41 | 0.32 | 0.21 |
| glucose | 0.69 | 0.36 | 0.39 | 0.38 |
| maltose | 0.73 | 0.40 | 0.38 | 0.42 |
| maltotriose | 0.73 | 0.39 | 0.39 | 0.42 |
| maltotetrose | 0.77 | 0.38 | 0.33 | 0.39 |
| maltopentose | 0.70 | 0.35 | 0.34 | 0.35 |

**Difference between the preventive effect of monomer, dimer and polymer of glucose.** One of them, monomer, dimer and polymer of glucose, was added to the protein solution at the concentration of 3.6 mg sugar per m*l* (20 mM with respect to glucose). Protein solubility, ATPase activity, numbers of SH-group and reduced viscosity were determined as shown in Table 3.

**Effect of polar group of cryoprotectant.** The significance of polar group, especially hydroxyl-group, of cryoprotective molecule had been already studied by NOGUCHI.[2] Then sorbitol, glucose, N-acetyl-D-glucosamine, N-1, 2, 6-acetyl-D-glucosamine and $\alpha$-pentaacetyl-D-glucose were added to the actomyosin solution at the concentration of 1.82, 1.80, 2.21, 3.15 and 3.90 mg/m*l* solution respectively. Results were shown in Table 4.

Table 4. Effect of polar group of cryoprotectants on the prevention of denaturation of actomyosin.

Protein Solubility

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 100% | 52.5 | 46.7 | 33.4 |
| sorbitol | | 61.1 | 54.2 | 54.6 |
| glucose | | 61.1 | 54.6 | 54.6 |
| N-acetyl glucosamine | | 57.8 | 52.8 | 42.5 |
| 1, 2, 6-acetyl glucosamine | | 60.1 | 50.4 | 41.2 |
| pentaacetyl glucose | | 60.1 | 45.7 | 34.6 |

ATPase Activity

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 100% | 84.9 | 51.3 | 20.3 |
| sorbitol | | 93.5 | 88.7 | 48.1 |
| glucose | | 120.9 | 91.6 | 45.2 |
| N-acetyl glucosamine | | 93.5 | 93.5 | 46.9 |
| 1, 2, 6-acetyl glucosamine | | 94.5 | 68.6 | 23.5 |
| pentaacetyl glucose | | 94.1 | 48.1 | 12.3 |

Numbers of SH-group

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 100% | 54.3 | 56.1 | 53.4 |
| sorbitol | | 62.4 | 62.9 | 61.2 |
| glucose | | 62.8 | 59.8 | 61.2 |

| | | | |
|---|---|---|---|
| N-acetyl glucosamine | 55.0 | 62.1 | 58.0 |
| 1, 2, 6-acetyl glucosamine | 59.9 | 57.9 | 44.3 |
| pentaacetyl glucose | 58.6 | 52.8 | 50.6 |

Reduced Viscosity

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 0.50 | 0.25 | 0.25 | 0.14 |
| sorbitol | 0.51 | 0.28 | 0.26 | 0.27 |
| glucose | 0.50 | 0.27 | 0.26 | 0.26 |
| N-acetyl glucosamine | 0.51 | 0.28 | 0.25 | 0.23 |
| 1, 2, 6-acetyl glucosamine | 0.51 | 0.28 | 0.28 | 0.17 |
| pentaacetyl glucose | 0.51 | 0.24 | 0.26 | 0.14 |

**Structure of cryoprotectant.** In order to investigate that whether hydroxyl group affected directly or not, inositol of hexavalent alcohol which was similar to the sorbitol having six hydroxyl groups was added to the actomyosin. Each additives was added at the concentration of 20 mM. Results were shown in Table 5.

Table 5. Effect of structure of cryoprotectants on the prevention of denaturation of actomyosin.

Protein Solubility

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 100% | 52.9 | 48.0 | 35.3 |
| glucose | | 57.5 | 56.2 | 43.4 |
| sorbitol | | 59.7 | 53.8 | 40.6 |
| inositol | | 53.8 | 48.8 | 31.9 |

ATPase Activity

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 100% | 55.3 | 105.2 | 44.5 |
| glucose | | 77.8 | 106.2 | 99.5 |
| sorbitol | | 76.1 | 111.6 | 99.5 |
| inositol | | 71.1 | 102.4 | 52.5 |

Numbers of SH-Group

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 100% | 93.8 | 84.4 | 85.8 |
| glucose | | 98.1 | 92.9 | 94.8 |
| sorbitol | | 100.9 | 99.9 | 94.3 |
| inositol | | 95.9 | 91.3 | — |

Reduced Viscosity

| Materials Added | Weeks | | | |
|---|---|---|---|---|
| | 0 | 2 | 4 | 8 |
| none | 0.68 | 0.30 | 0.27 | 0.20 |
| glucose | 0.69 | 0.32 | 0.34 | 0.33 |
| sorbitol | 0.68 | 0.30 | 0.36 | 0.36 |
| inositol | 0.68 | 0.34 | 0.31 | 0.22 |

## Discussion

The cryoprotective effect of glucose was increased in proportion to the concentration of glucose. With regard to protein solubility, glucose was most effective at the concentration of 0.2 mM. High concentration of glucose (20 mM) was not so effective as expected. ATPase activity was well kept during storage at the high concentration of glucose. With regard to thiol-group, the increase of numbers of thiol-group by addition of glucose at high concentration seemed due to the reducing power by glucose.

As shown in Table 3, it was found that maltotriose was most effective on ATPase activity, numbers of thiol-group and solubility of actomyosin during frozen storage. This result agreeds with the report on the protective effect of polyethylene glycols on denaturation of actomyosin by NIWA *et al.*[7]

With respect to reduced viscosity, the relation between preventive effect and molecular weight of cryoprotectant was almost same as result with respect to the solubility.

As shown in Table 4, it was found that numbers of hydroxyl group was important for the cryoprotection of sugar. Protein solubility and reduced viscosity were increased in proportion to the numbers of hydroxyl group of cryoprotectant. N-acetyl-D-glucosamine revealed almost same effect as glucose and sorbitol protected ATPase activity. However $\alpha$-pentaacetyl-D-glucose which has no hydroxyl group inhibited ATPase activity.

The preventive effect of inositol was inferior than that of sorbitol. It was seemed

that the difference between sorbitol and inositol in structure (molecular form) affected the preventive effect during frozen storage of protein (Table 5).

## References

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